



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C09B 67/00, A61K 7/13</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 97/19999</b> <b>(43) International Publication Date:</b> 5 June 1997 (05.06.97)
<b>(21) International Application Number:</b> PCT/DK96/00499 <b>(22) International Filing Date:</b> 29 November 1996 (29.11.96) <b>(30) Priority Data:</b> 1357/95 30 November 1995 (30.11.95) DK <b>(71) Applicant (for all designated States except US):</b> NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> AASLYNG, Dorrit [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). SØRENSEN, Niels, Henrik [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). RØRBÆK, Karen [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). <b>(74) Common Representative:</b> NOVO NORDISK A/S; Novo Allé, DK-2880 Bagsværd (DK).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> LACCASES WITH IMPROVED DYEING PROPERTIES <b>(57) Abstract</b> <p>The present invention relates to a permanent dyeing composition comprising: a) above 0 to 1 mg enzyme protein per ml dyeing composition of microbial laccase, b) one or more dye precursor, and c) optionally one or more dye modifiers, the use of the dyeing composition for dyeing keratinous fibres, such as hair, fur, hide and wool, and a method for permanent dyeing of keratinous fibres.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

Titl : Laccases with improved dyeing properties

#### FIELD OF THE INVENTION

5 The present invention relates to a dyeing composition comprising a microbial laccase, the use of said dyeing composition for dyeing keratinous fibres, in particular hair, fur, hide and wool, and a method for dyeing keratinous fibres.

#### 10 BACKGROUND OF THE INVENTION

It has been used for many years to dye the hair to cover appearing grey hair. The need to do so arises from the fact that grey hair is the first sign of having past adolescence, which can be hard to accept for many people.

15 For instance, in certain parts of Asia it is widely used by both men and women to dye the hair with dyes often referred to by humorous people as "black shoe polish".

During the last few decades hair dyeing has become more and more popular in the western world. At first Punk Rockers and  
20 other society critical groups dyed their hair in extreme colours as a part of their protest against the established society, but today especially many young people also uses hair dyes (in more soft tints than the Punk Rockers) as a sort of "cosmetic" to change or freshen up their "look".

25

#### Hair dyes

In general hair dyeing compositions on the market today can be divided into three main groups:

- temporary hair dyes,
- 30 - semi-permanent hair dyes, and
- permanent oxidative hair dyes.

The temporary hair dyes are only intended to change the natural hair colour for a short period of time and usually functions by depositing dyes on the surface of the hair. Such  
35 hair dyes are easy to remove with normal shampooing.

When using semi-permanent hair dyes the colour of the dyed hair can survive for five or more shampoos. This is achieved

by using dyes having a high affinity for hair keratin and which is able penetrate into the interior of the hair shaft.

Permanent hair dyes are very durable to sunlight, shampooing and other hair treatments and need only to be refreshed once a month as new hair grows out. With these dyeing systems the dyes are created directly in and on the hair. Small aromatic colourless dye precursors (e.g. p-phenylene-diamine and o-aminophenol) penetrate deep into the hair where said dye precursors are oxidised by an oxidising agent into coloured polymeric compounds. These coloured compounds are larger than the dye precursors and can not be washed out of the hair.

By including compounds referred to as modifiers (or couplers) in the hair dyeing composition a number of hair colour tints can be obtained. Cathecol and Resorcinol are examples of such modifiers.

Traditionally  $H_2O_2$  is used as the oxidizing agent (colour builder), but also as a bleaching agent. Dyeing compositions comprising  $H_2O_2$  are often referred to as "lightening dyes" due to this lightening effect of  $H_2O_2$ .

The use of  $H_2O_2$  in dyeing compositions have some disadvantages as  $H_2O_2$  damages the hair. Further, oxidative dyeing often demands high pH (normally around pH 9-10), which also inflicts damage on the hair and on the skin. Consequently, if using dye compositions comprising  $H_2O_2$  it is not recommendable to dye the hair often.

To overcome the disadvantages of using  $H_2O_2$  it has been suggested to use oxidation enzymes to replace  $H_2O_2$ .

US patent no. 3,251,742 (Revlon) describes a method for dyeing human hair by dye formation *in situ* (i.e. on the hair). An oxidative enzyme is used to the colour formation reactions at a substantially neutral pH (7-8.5). Laccases, tyrosinases, polyphenolases and catacolases are mentioned as suitable oxidation enzymes. The only exemplified oxidation enzyme is tyrosinase.

EP patent no. 504.005 (Perma S.A.) concerns dyeing compositions for keratinous fibres, in particular hair, which do not require the presence of  $H_2O_2$  (hydrogen peroxide). The

composition comprises an enzyme capable of catalysing the formation of the polymeric dyes and also dye precursors, such as bases and couplers, in a buffer solution wherein the pH of said composition is between 6.5 and 8 and said enzyme has an  
5 optimal activity in the pH range between 6.5 and 8.

*Rhizoctonia praticola* laccase and *Rhus vernicifera* laccase are the only laccases exemplified in the patent.

Abstract of Papers American Chemical Society vol. 209, no. 1-2, 1995 discloses the cloning of laccases from *Scytalidium*  
10 *thermophilum* and *Myceliophthora thermophila*. The abstract does not mention the use of said laccases for dyeing.

#### SUMMARY OF THE INVENTION

The object of the present invention is to provide improved  
15 dyeing compositions for keratinous fibres, such as hair, fur hide and wool, comprising an oxidative enzyme as the oxidising agent.

In the context of the present invention an "improved" composition for dyeing keratinous fibres means a composition  
20 being capable of dyeing the keratinous fibres in question faster or by the use of a smaller amount of oxidation enzyme to obtain an optimal dyeing effect, determined as  $\Delta E^*$ , in comparison to corresponding prior art dyeing compositions.

Further, it is also possible to use a less amount of dye  
25 precursor. This is advantageous as certain dye precursors are very unhealthy and very carcinogenic.

Compositions capable of dyeing the keratinous fibres, in particular hair, fur, hide and wool, faster are desirable as such compositions are very user convenient.

30 Further, it is desirable to be able to use a less amount of enzyme in the dyeing composition. This might make the dyeing process more economical. Further, the risk for creating airborne protein aerosols is reduced.

It has now surprisingly been found that it is possible to  
35 provide such improved dyeing compositions for keratinous fibres by using microbial laccases for oxidising the dye precursor(s).

Laccases (benzenediol:oxygen oxidoreductases) (E.C. class

1.10.3.2 according to Enzyme Nomenclature (1992) Academic Press, Inc.) are multi-copper containing enzymes that catalyse the oxidation of phenols. Laccase-mediated oxidation results in the production of aryloxy-radical intermediates from suitable phenolic substrates; the ultimate coupling of the intermediates so produced provides a combination of dimeric, oligomeric, and polymeric reaction products. Certain reaction products may be used to form dyes suitable for dyeing keratinous fibres, such as hair and wool (see below).

10 Firstly, the object of the invention is to provide a dyeing composition comprising

- a) above 0 to 1 mg enzyme protein per ml dyeing composition of microbial laccase,
- b) one or more dye precursors,
- 15 c) optionally one or more modifiers.

Specifically contemplated is laccases of microbial origin, derived from a strain of the genus *Myceliophthora*.

In the second aspect the invention relates to the use of a dyeing composition of the invention for dyeing keratinous fibres, such as hair, fur, hide and wool.

20 The invention also related to a method for dyeing keratinous fibres comprising contacting the dyeing composition of the invention to the keratinous fibres in question under suitable conditions and for a period of time sufficient to permit oxidation of the dye precursor into a coloured compound.

25 The invention also relates to the use of a laccase for permanent dyeing of keratinous fibres wherein said laccase is a laccase that results in a  $\Delta E^*$ -value higher than the  $\Delta E^*$ -value resulting from a laccase derived from *Rhus* under corresponding dyeing conditions.

30 This means that when dyeing keratinous fibres with a dyeing composition of the present invention the  $\Delta E^*$ -value determined are higher than the  $\Delta E^*$ -value determined from corresponding keratinous fibres dyed under the same conditions using a dyeing composition comprising a laccase derived from *Rhus*.

35 The term "corresponding dyeing conditions" means under conditions where e.g. the enzyme concentration or enzyme

activity, dyeing incubations time, dyeing incubation temperature, pH conditions, keratin fibre type (such as hair type) are the same, and further that the same dye precursor(s) and modifier(s) are used. In other words it defines conditions parallel to the specific dyeing conditions chosen. The dyeing conditions described below in the Examples may be chosen.

In the context of the present invention a "higher"  $\Delta E^*$  value defines that the total quantitative colour change is more than one  $\Delta E^*$  unit.

$\Delta E^*$  is calculated from the values of the parameters  $a^*$ ,  $b^*$  and  $L^*$  determined e.g. on a Minolta CR200 Chroma Meter using the formula  $\Delta E^* = \sqrt{(\Delta L^*^2 + \Delta a^*^2 + \Delta b^*^2)}$ . The meaning of  $a^*$ ,  $b^*$  and  $L^*$  is explained below in the "Materials and Methods" section.

#### BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows the dyeing effect of six different laccases. The six laccases are the *Polyporus pinsitus* laccase (rPp-laccase), *Myceliophthora thermophila* laccase (Mt-laccase wt.), *Myceliophthora thermophila* T1 variant laccase (Mt-laccase (var)), *Rhus vernicifera* laccase (Rvl-FXu-1), *Scytalidium thermophilum* laccase (rStL-FXu-1) and *Rhizoctonia solani* laccase (rRSL-3-FXu-1). o-aminophenol is used as the dye precursor and m-phenylene-diamine is used as a modifier.

Figure 2 shows the wash stability of the *Myceliophthora thermophila* T1 variant laccase (Mt-laccase (var)) and the *Polyporus pinsitus* laccase (rPp-laccase) as the oxidising agent.

Figure 3 shows the fastness (speed) of hair dyeing using the *Myceliophthora thermophila* T1 variant laccase (Mt-laccase (var)) and the *Polyporus pinsitus* laccase (rPp-laccase) as the oxidising agent.

Figure 4 shows the dose-response dyeing effect of *Myceliophthora thermophila* laccase, using from 0.0001 to 0.5 mg enzyme protein per ml dyeing composition.

**DETAILED DESCRIPTION OF THE INVENTION**

The object of the present invention is to provide improved dyeing compositions for permanent dyeing of keratinous fibres, such as hair, fur, hide and wool, comprising an oxidation  
5 enzyme.

It has now surprisingly been found that it is possible to provide such improved dyeing compositions by using a microbial laccase for oxidising the dye precursor(s).

10 The Dyeing Composition

In the first aspect the present invention relates to a dyeing composition comprising

- a) above 0 to 1 mg enzyme protein per ml dyeing composition of microbial laccase,
- 15 b) one or more dye precursor, and
- c) optionally one or more dye modifiers.

In a preferred embodiment of the invention the laccase may be present in the dyeing compositions in a concentration within the range from 0.0001 to 1 mg/ml, preferably 0.001 to 0.8  
20 mg/ml, more preferred 0.002 to 0.5, even more preferred 0.003 to 0.2, especially 0.004 to 0.1 mg enzyme protein/ml dyeing composition.

When dyeing with a composition of the invention for permanent dyeing the  $\Delta E^*$ -value obtained is higher than that  
25 obtained when using a dyeing composition comprising a laccase derived from *Rhus* under corresponding dyeing conditions.

An example of a *Rhus* laccase is the laccase derived from the Japanese varnish tree *Rhus vernicifera* (Yoshida, (1883), J. Chem. Soc., 472). The *Rhus vernicifera* laccase is used in the  
30 Example 1 below.

The microbial laccase used according to the invention is of fungal or bacteria origin, in particular of filamentous fungus origin.

In an embodiment of the invention the microbial laccase is  
35 derived from a strain of genus *Myceliophthora*, such as a strain of the species *Myceliophthora thermophila* e.g. the purified laccase described in WO 95/33836 (PCT/US95/06815) from Novo



Nordisk, which is hereby incorporated by reference. SEQ ID NO 1 below shows a DNA sequence encoding a suitable laccase derivable from *Myceliophthora thermophila*.

5 *E. coli* JM101 containing the expression vector pRAMB5 comprising SEQ ID NO 1 has been deposited under the Budapest Treaty with the Agricultural Research Service Patent Culture Collection, Northern Regional Research Center, 1815 University Street, Peoria, Illinois, 61604. The vector have been given the Accession Number NRRL B-21261.

10 Also contemplated according to the invention are laccases derived from other micro-organisms being more than 80% homologous to SEQ ID NO 1 derived from *Myceliophthora thermophila*.

In addition, *Myceliophthora* laccases also encompass alternative forms of laccases which may be found in *M. thermophila* and  
15 as well as laccases which may be found in other fungi which are synonyms of fall within the definition of *M. thermophila* as described by Apinis (Nova Hedwigia 5, 57-78, 1963) and named *Sporotrichum thermophile*. Subsequent taxonomic revisions have placed this organism in the genus *Chrysosporium* (Von Klopotek,  
20 A. Arche., (1974) Microbiol, 98, 365-369) and later *Myceliophthora* (Van Oorshot, Persoonia, (1977), 9, 401-408). A number of organisms known by other named also appear to belong to this species. These include *Sporotrichum cellulophilum* (US patent no. 4,106,989); *Thielavia thermophila* (Fergus and Sinden  
25 (1968), Can. J. Botany, 47, 1635-1637); *Chrysosporium fergusi* and *Corynascus thermophilus* (Von Klopotek, supra).

Also the use of laccase variants are contemplated according to the present invention.

An example of a laccase variant is the *Myceliophthora thermophila* T1 variant described in PCT/US96/14087 (Novo Nordisk).  
30

T1 variants (or Type I variants) are modified blue copper oxidases, including laccases. T1 variants can for instance be constructed by site-directed mutagenesis and differ from the corresponding wild-type blue copper oxidases by at least one  
35 amino acid residue in the Type I (T1) copper site. These modifications generally result in altered pH profiles and/or specific activity relatively to the wild-type enzymes. This can

be advantageous when using the enzyme in question in dyeing compositions.

More specific the *Myceliophthora thermophila* T1 laccase variant may comprise the sequence 509VSGGL511 or may be  
5 modified as to increase the negative charge in at least one segment of the T1 copper site.

The above mentioned microbial laccases may advantageously be used in permanent dyeing composition for keratinous fibres. Such compositions have a superior dyeing effect to  
10 corresponding compositions comprising e.g. the *Rhus vernicifera* laccase as shown in Example 1.

The *Myceliophthora thermophila* T1 variant laccase is more wash stable and further dyes faster than the *Polyporus pinsitus* laccase which is proven in Example 2 and Example 3, respectively.  
15

Example 4 shows that less *Myceliophthora* laccase activity (i.e. LACU/ml) is needed to obtain a suitable dyeing effect in comparison to the *Polyporus pinsitus* laccase.

Example 5 shows that for the *Myceliophthora thermophila* laccase the dyeing effect optimum is obtained around 0.005 mg  
20 enzyme protein per ml dyeing composition.

In the case of using a *Myceliophthora* laccase in a permanent dyeing composition it may advantageously be present in concentrations from above 0 to 1 mg/ml, preferably 0.0001 to  
25 0.1 mg/ml, more preferably 0.0005 to 0.05 mg/ml, especially 0.001 to 0.01 mg enzyme protein per ml dyeing composition.

It is to be understood that the laccase may be produced either homologously, or heterologously in a host cell such as filamentous fungus, yeast or bacteria.

30 Examples of filamentous fungi host cells include strains of the species of *Trichoderma*, preferably a strain of *Trichoderma harzianum* or *Trichoderma reesei*, or a species of *Fusarium*, or a species of *Aspergillus*, most preferably *Aspergillus oryzae* or *Aspergillus niger*, or yeast cells, such as e.g. a strain of  
35 *Saccharomyces*, in particular *Saccharomyces cerevisiae*, *Saccharomyces kluyveri* or *Saccharomyces uvarum*, a strain of *Schizosaccharomyces* sp., such as *Schizosaccharomyces pombe*, a

strain of *Hansenula* sp., *Pichia* sp., *Yarrowia* sp., such as *Yarrowia lipolytica*, or *Kluyveromyces* sp., such as *Kluyveromyces lactis*, or a bacteria, such as gram-positive bacteria such as strains of *Bacillus*, such as strains of *B. subtilis*, *B. Licheniformis*, *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. coagulans*, *B. circulans*, *B. lautus*, *B. megaterium* or *B. thuringiensis*, or strains of *Streptomyces*, such as *S. lividans* or *S. murinus*, or gram-negative bacteria such as *Escherichia coli*.

10 To obtain dyeing of the keratinous fibres the dyeing composition of the invention comprises one or more dye precursors which is(are) converted into coloured compound(s) by an oxidation agent which according to the present invention is a microbial laccase.

15 Without being limited thereto the dye precursor(s) may be (an) aromatic compound(s) belonging to one of three major chemical families: the diamines, aminophenols (or aminonaphtols) and the phenols. Examples of isatin derivative dye precursors can be found in DE 4,314,317-A1. Further, a number  
20 of indole or indoline derivative dye precursors are disclosed in WO 94/00100. Said dye precursors mentioned in these documents are hereby incorporated herein by reference.

Examples of suitable dye precursors include compounds from the group comprising p-phenylene-diamine (PPD), p-toluylene-diamine (PTD), chloro-p-phenylene-diamine, p-aminophenol, o-aminophenol, 3,4-diaminotoluene, 2-methyl-1,4-diaminobenzene, 4-methyl-o-phenylenediamine, 2-methoxy-p-phenylenediamine, 2-chloro-1,4-diamino-benzene, 4-amino diphenylamine, 1-amino-4- $\beta$ -methoxyethylamino-benzene, 1-amino-4-bis-( $\beta$ -hydroxyethyl)-aminobenzene, 1-3-diamino-benzene, 2-methyl-1,3-diamino-benzene,  
30 2,4-diaminotoluene, 2,6-diaminopyridine, 1-hydroxy-2-amino-benzene, 1-hydroxy-3-amino-benzene, 1-methyl-2-hydroxy-4-amino-benzene, 1-methyl-2-hydroxy-4- $\beta$ -hydroxyethylamino-benzene, 1-hydroxy-4-amino-benzene, 1-hydroxy-4-methylamino-benzene, 1-methoxy-2,4-diamino-benzene, 1-ethoxy-2,3-diamino-benzene, 1- $\beta$ -hydroxyethyloxy-2,4-diamino-benzene, phenazines, such as 4,7-

phenazinedicarboxylic acid, 2,7-phenazinedicarboxylic acid, 2-phenazinecarboxylic acid, 2,7-diaminophenazine, 2,8-diaminophenazine, 2,7-diamino-3,8-dimethoxyphenazine, 2,7-diamino-3-methoxyphenazine, 2,7-diamino 3-methoxyphenazine, 3-dimethyl

5 2,8-phenazinediamine, 2,2'-[(8-amino-7-methyl-2-phenazinylo)imino]bis-ethanol, 2,2'-[(8-amino-7-methoxy-2-phenazinylo)imino]bis-ethanol, 2,2'-[(8-amino-7-chloro-2-phenazinylo)imino]bis-ethanol, 2-[(8-amino-7-methyl-2-phenazinylo)amino]-ethanol, 2,2'-[(8-amino-2-phenazinylo)imino]bis-ethanol, 3-amino-7-(dime-

10 thylamino)-2,8-dimethyl-5-phenyl-chloride, 9-(diethylamino)-benzo[a]phenazine-1,5-diol, N-[8-(diethylamino)-2-phenazinylo]-methanesulfonamide, N-(8-methoxy-2-phenazinylo)-Methanesulfonamide, N,N,N',N'-tetramethyl-2,7-phenazinediamine, 3,7-dimethyl-2-phenazinamine, p-amino benzoic acids, such as p-amino

15 benzoic acid ethyl, p-amino benzoic acid glycerid, p-amino benzoic acid isobutyl, p-dimethylamino benzoic acid amil, p-dimethylamino benzoic acid octyl, p-diethoxy amino benzoic amil, p-dipropoxy amino benzoic acid ethyl, acetylsalicylic acid, isatin derivatives, such as 2,3-diamino benzoic acid.

20 In an embodiment the laccase is used with the dye precursor directly to oxidise it into a coloured compound. The dye precursor may be used alone or in combination with other dye precursors.

It is believed that when using a diamine or an aminophenol

25 as the dye precursor at least one of the intermediates in the co-polymerisation must be an *ortho*- or *para*-diamine or aminophenol. Examples of such are found below and are also described in US patent no. 3,251,742 (Revlon), the contents of which are incorporated herein by reference.

30 Optionally dyeing compositions (especially hair dyeing compositions) of the invention also comprise a modifier (coupler) by which a number of colour tints can be obtained.

In general modifiers are used in dyeing composition for hair as the hair colours resulting from hair dyeing compositions

35 without modifier(s) are usually unacceptable for most people.

Modifiers are typically *m*-diamines, *m*-aminophenols, or polyphenols. Upon the optional addition of a modifier (coupler) it

reacts with the dye precursor(s) in the presence of e.g. a laccase, converting the dye precursor(s) into a coloured compound.

Examples of modifiers (couplers) include m-phenylene-diamine, 2,4-diaminoanisole, 1-hydroxynaphthalene( $\alpha$ -naphthol), 1,4-dihydroxybenzene(hydroquinone), 1,5-dihydroxynaphthalene, 1,2-dihydroxybenzene(pyrocatechol), 1,3-dihydroxybenzene (resorcinol), 1,3-dihydroxy-2-methylbenzene, 1,3-dihydroxy-4-chlorobenzene(4-chlororesorcinol), 1,2,3-trihydroxybenzene, 1,2,4-trihydroxybenzene, 1,2,4-trihydroxy-5-methylbenzene, 1,2,4-trihydroxytoluene.

When using the dyeing compositions of the invention a reduced amount of enzyme (i.e. mg enzyme protein per ml dyeing composition) is needed to obtain the maximal dyeing effect (See Figure 1 and Figure 4), determined as the optimal  $\Delta E^*$ -value, in comparison to prior art dyeing compositions, such as dyeing compositions comprising a laccase derived from *Rhus*.

The amount of dye precursor(s) and other ingredients used in the composition of the invention are in accordance with usual commercial amounts.

According to the invention the product comprising the dyeing composition may be a one or a two compartment product. In the one compartment product the laccase, the dye precursor(s) and other ingredients are kept together in a stabilised solution or kept under stable conditions (i.e. the dye precursors are not oxidised by the laccase). In a two compartment product the laccase and the dye precursor(s) and other ingredients are kept in two containers kept apart. The contents of said containers are mixed immediately before use.

#### 30 USE

In the second aspect the invention relates to the use of the dyeing composition of the invention for permanent dyeing of keratinous fibres, in particular hair, fur, hide and wool.

When using a dyeing composition of the invention the  $\Delta E^*$ -value obtained is higher than that of a dyeing composition comprising a laccase derived from genus *Rhus* under corresponding dyeing conditions (see Figure 1).

### Method

In the third aspect the invention relates to a method for permanent dyeing of keratinous fibres comprising contacting a dyeing composition of the invention with the keratinous fibres  
5 in question under suitable conditions and for a period of time sufficient to permit oxidation of the dye precursor into a coloured compound.

The dyeing procedure may be carried out at room temperature, preferably around the optimal temperature of the enzyme, typi-  
10 cally with from 10 to 60°C; at a pH in the range from 3 to 10, preferably 5 to 9, especially 6 to 8; for a period of time between 10 and 60 minutes, preferably 15 to 50 minutes, especially 20 to 40 minutes.

When using the method of the invention the  $\Delta E^*$ -value  
15 obtained is higher than that of corresponding methods where a laccase derived from a strain of the genus *Rhus* are used under the same dyeing conditions, in the presence or absence of at least one modifier, with at least one dye precursor, for a period of time, and under conditions sufficient to permit  
20 oxidation of the dye precursor used for oxidising the dye.

The method can be conducted with one or more dye precursors, either alone or in combination with one or more modifiers.

### **MATERIALS AND METHODS**

#### **25 Materials:**

##### Hair:

6" De Meo Virgin Natural White Hair (De Meo Brothers Inc. USA)

##### Enzymes:

*Myceliophthora thermophila* laccase described in WO 95/33836  
30 (PCT/US95/06815) from Novo Nordisk Biotech, Inc.

*Myceliophthora thermophila* T1 variant laccase described in US patent application 60/003,142 from Novo Nordisk Biotech, Inc.

*Polyporus pinsitus* laccase described in WO 96/00290  
(PCT/US95/07536) from Novo Nordisk Biotech, Inc.

35 *Rhus vernicifera* laccase (Yoshida, J. Chem. Soc., 472 (1883)

*Rhizoctonia solani* laccase described in WO 95/07988 from Novo Nordisk Biotech, Inc.

*Scytalidium thermophilum* laccase described in WO 95/33837 (PCT/US95/06816) from Novo Nordisk Biotech, Inc.

Deposit of Biological Material

5       The following biological material has been deposited on the  
25 May 1994 under the terms of the Budapest Treaty with the  
Agricultural Research Service Patent Culture Collection,  
Northern Regional Research Center, 1815 University Street,  
Peoria, Illinois, 61604 and given the following accession  
10       number.

Deposit

Accession Number

*E. coli* JM101 containing pRAMB5

NRRL B-21261

15       Dye precursors:

0.1 % w/w p-phenylene-diamine in 0.1 M K-phosphate buffer, pH  
7.0. (PPD)

0.1 % w/w p-toluylyene-diamine in 0.1 M K-phosphate buffer, pH  
7.0.

20       0.1 % w/w chloro-p-phenylenediamine in 0.1 M K-phosphate  
buffer, pH 7.0.

0.1 % w/w p-aminophenol in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % w/w o-aminophenol in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % w/w 3,4-diaminotoluene in 0.1 M K-phosphate, buffer pH  
25       7.0.

Modifiers:

0.1 % w/w m-phenylenediamine in 0.1 M K-phosphate buffer, pH  
7.0.

30       0.1 % w/w 2,4-diaminoanisoie in 0,1 M K-phosphate buffer, pH  
7.0.

0.1 % w/w alpha-naphthol in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % w/w hydroquinone in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % w/w pyrocatechol in 0.1 M K-phosphate buffer, pH 7.0.

35       0.1% w/w resorcinol in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % w/w 4-chlororesorcinol in 0.1 M K-phosphate buffer, pH  
7.0.

The dye precursor is combined with one of the above indicated modifiers so that the final concentration in the dyeing solution is 0.1 % w/w with respect to precursor and 0.1 % w/w with respect to modifier.

5

Other solutions:

Commercial shampoo

Equipment:

10 Minolta CR200 Chroma Meter

Determination of Laccase Activity (LACU)

Laccase activity is determined from the oxidation of syringaldazin under aerobic conditions. The violet colour produced is photometered at 530 nm. The analytical conditions are 19 mM syringaldazin, 23.2 mM acetate buffer, pH 5.5, 30°C, 1 min. Reaction time.

1 laccase unit (LACU) is the amount of enzyme that catalyses the conversion of 1.0 micromole syringaldazin per minute at these conditions.

20

Assessment of the hair colour

The quantitative colour of the hair tresses is determined on a Minolta CR200 Chroma Meter by the use the parameters L\* ("0"=black and "100"=white), a\* ("-"=green and "+"=red) and b\* ("-" blue and "+" yellow).

25

$\Delta L^*$ ,  $\Delta a^*$  and  $\Delta b^*$  are the delta values of L\*, a\* and b\* respectively compared to L\*, a\* and b\* of untreated hair (e.g.  $\Delta L^* = L^*_{\text{sample}} - L^*_{\text{untreated hair}}$ ).

30  $\Delta E^*$  is calculated as  $\Delta E^* = \sqrt{(\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})}$  and is an expression for the total quantitative colour change.

**EXAMPLES**

35 **Example 1**

Dyeing effect



The dyeing effect of different laccases were tested and compared under the same conditions using 0.1% w/w o-aminophenol (dye precursor) and 0.1% w/w m-phenylene-diamine (modifier).

The laccases tested were

- 5 a *Polyporus pinsitus* laccase,
- a *Myceliophthora thermophila* laccase
- a *Myceliophthora thermophila* T1 laccase variant,
- a *Rhus vernicifera* laccase
- a *Rhizoctonia solani* laccase
- 10 a *Scytalidium thermophila* laccase

#### Hair dyeing

1 gram white De Meo hair tresses were used.

- 4 ml dye precursor solution (including modifier) was mixed
- 15 with 1 ml laccase on a Whirley mixer, applied to the hair tresses and incubated at 30°C for 60 minutes.

The hair tresses were then rinsed with running water, washed with shampoo, rinsed with water, combed, and air dried.

- a\*, b\* and L\* were determined on the Chroma Meter and  $\Delta E^*$
- 20 was then calculated.

Hair tress samples treated without enzyme were used as a blind.

The result of the test is displayed in figure 1.

#### 25 **Example 2**

##### Wash stability

- Tresses of white De Meo hair (1 gram) were used for testing
- of the wash stability of hair dyed using the *Myceliophthora thermophila* T-variant laccase and the *Polyporus pinsitus*
- 30 laccase.

Oxidative hair dyeing was carried out as described in Example 1, except that p-phenylene-diamine (PPD) were used as the dye precursor and no modifiers were used.

#### 35 Hair wash

The dyed hair tresses were wetted and washed for 15 seconds with 50 ml of commercial shampoo, and rinsed with water for 1

minute and air dried. The hair tresses were washed up to 18 times.

Then  $a^*$ ,  $b^*$  and  $L^*$  were determined on the Chroma Meter and  $\Delta E^*$  values were then calculated.

5 Hair tress samples treated without enzymes were used as blinds.

The result of the test is displayed in figure 2.

### Example 3

#### 10 Fastness of hair dyeing

Tresses of white De Meo hair (1 gram) were used for testing fastness (speed) of hair dyeing using the *Myceliophthora thermophila* T1 variant laccase and the *Polyporus pinsitus* laccase.

15 p-phenylene-diamine (PPD) was used as the dye precursor and no modifiers were used.

4 ml dye precursor solution was mixed with 1 ml laccase on a Whirley mixer, applied to the hair tresses and incubated at 30°C for 10, 20, 30, 40, 50 and 60 minutes, respectively.

20 The hair tresses were then rinsed with running water, washed with shampoo, rinsed with running water, combed, and air dried.

$a^*$ ,  $b^*$  and  $L^*$  were determined on the Chroma Meter for each incubation time and the  $\Delta E^*$ -values were then calculated.

Hair tress samples treated without enzymes for 60 minutes  
25 were used as blinds.

The result of the test is displayed in figure 3.

### Example 4

#### Dyeing effect of *Myceliophthora thermophila* T1 variant laccase

30 The dyeing effect of *Myceliophthora thermophila* T1 variant laccase were compared with the *Polyporus pinsitus* laccase using 0.1% w/w p-phenylene-diamine, 0.1% w/w p-touylene-diamine, 0.1% w/w chloro-p-phenylene-diamine, 0.1% w/w p-aminophenol, 0.1% w/w o-aminophenol and 0.1% w/w 3,4 diaminotoluene, respectively,  
35 ly, as dye precursors.

The *Polyporus pinsitus* laccase were applied in a concentration of 10 LACU/ml while the *Myceliophthora*

*thermophila* T1 variant laccase was applied in a concentration of only 1 LACU/ml.

1 gram white De Meo hair tresses were used.

4 ml dye precursor solution was mixed with 1 ml laccase on a  
5 Whirley mixer, applied to the hair tresses and incubated at 30°C for 60 minutes.

The hair tresses were then rinsed with running water, washed with shampoo, rinsed with running water, combed, and air dried.

The a\*, b\* and L\* were determined on the Chroma Meter and  
10 the  $\Delta E^*$  values were then calculated.

Hair tress samples treated without enzyme were used as blinds.

The result of the test is displayed in Table 1.

15 Table 1

Sample	<i>Polyporus pinsitus</i> laccase $\Delta E^*$	<i>Myceliophthora thermophila</i> T1 variant laccase $\Delta E^*$
p-phenylene-diamine blind	9.7	10.9
p-phenylene-diamine + laccase	52.7	52.9
p-toluylene-diamine blind	16.1	18.6
p-toluylene-diamine + laccase	39.1	38.2
chloro-p-phenylene-diamine blind	2.6	4.0
chloro-p-phenylene-diamine + laccase	40.5	39.2
p-aminophenol blind	6.2	7.0
p-aminophenol + laccase	32.4	28.1
o-amonophenol blind	5.6	6.4
o-amonophenol + laccase	22.9	22.0
3,4-diaminotoluene blind	3.4	2.6
3,4-diaminotoluene + laccase	36.5	42.2

As can be seen from Tabl 1 compositions comprising the *Myceliophthora thermophila* T1 laccase variant dyes the hair as good as the *Polyporus pinsitus* laccase even though

concentration of the *Polyporus pinsitus* laccase is 10 time higher.

Example 5

5 Dose-response dyeing effect of *M. thermophila* laccase

The dyeing effect of *M. thermophila* laccase were tested using concentration between 0.0001 to 0.5 mg enzyme protein per ml dyeing composition of laccase. 0.1% w/w p-toluylene-diamine (PTD) was used as the dye precursor.

- 10 The same dyeing procedure as described in Example 1 was used. The result of the tests are displayed in Figure 4.

## SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Novo Nordisk A/S  
 (B) STREET: Novo Alle  
 (C) CITY: Bagsvaerd  
 (D) COUNTRY: Denmark  
 (E) POSTAL CODE (ZIP): DK-2880  
 (F) TELEPHONE: +45 4444 8888  
 (G) TELEFAX: +45 4449 3256

(ii) TITLE OF INVENTION: Laccases with improved dyeing properties

(iii) NUMBER OF SEQUENCES: 2

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
 (B) COMPUTER: IBM PC compatible  
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3192 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS  
 (B) LOCATION: join(586..831, 917..994, 1079..1090, 1193..1264, 1337..2308, 2456..2524, 2618..3028)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCTAGCTTCT TTGGTCACCG TCGTTTTTCGC CCGCCCCCTC CCTCCTTCAA CCCCCTGAGT	60
AGTCGGCTAA GCGATCCTCA ATCTGGTCTT GTGAGGTCAC GTCCTCCAGC AGATGACAGT	120
TCATCGAGCG AGTGATCTCC ACCACCCAGA AGGGAGGGGG GATGCGCGCA TGCTCCAACA	180
TCCCTGGTGT CGCTAGAGAC GTCGCGGCAT CAGCCTTTTC ATCACACCGA GCACGTCCAC	240
GGACCGGCTC CTTTCACCCC CGCGTCTCTC GGAGGATTGA GTCACGATAT TTCGGGATGT	300
GGGAAGGGGG AGAGAAAGGA GGGGGGAGGG GCGGAAACAT GTTGATACG AGCTGCGCCC	360
CTTTTTCAAC ATCGAGAACA GGAAGTCGTT GGTGTCGGCC GTAATGTCTA TAAACGAGG	420
CTCCTTCTCG TCGTCGACTT GTCTCAGGTT CTCTCTCTCG TCCACACCAA GCCAGTCTTG	480
CCTGAGCCAC CTGAGCCACC TTCAACTCAT CATCTTCAGT CAAGTCGTTT ATTGACATTG	540
TGTCTCTCTT TCTATCGAGT CGGCTTCCCG GCCCTTCACC ACAAC ATG AAG TCC	594
	Met Lys Ser
	1
TTC ATC AGC GCC GCG ACG CTT TTG GTG GGC ATT CTC ACC CCT AGC GTT	642
Phe Ile Ser Ala Ala Thr Leu Leu Val Gly Ile Leu Thr Pro Ser Val	
5 10 15	

20

	GCT GCT GCC CCT CCA TCC ACC CCT GAG CAG CGC GAC CTG CTC GTC CCG	690
	Ala Ala Ala Pro Pro Ser Thr Pro Glu Gln Arg Asp Leu Leu Val Pro	
	20 25 30 35	
5	ATC ACG GAG AGG GAG GAG GCA GCC GTG AAG GCT CGC CAG CAG AGC TGC	738
	Ile Thr Glu Arg Glu Glu Ala Ala Val Lys Ala Arg Gln Gln Ser Cys	
	40 45 50	
10	AAC ACC CCC AGC AAC CGG GCG TGC TGG ACT GAC GGA TAC GAC ATC AAC	786
	Asn Thr Pro Ser Asn Arg Ala Cys Trp Thr Asp Gly Tyr Asp Ile Asn	
	55 60 65	
15	ACC GAC TAC GAA GTG GAC AGC CCG GAC ACG GGT GTT GTT CCG CCG	831
	Thr Asp Tyr Glu Val Asp Ser Pro Asp Thr Gly Val Val Arg Pro	
	70 75 80	
	GTGAGTGCTC TCGTTAATTA CGCTTCGGCG AGTTGCCGAG ATATATTAAA TACTGCAAAC	891
20	CTAAGCAGGA GCTGACATGC GACAG TAC ACT CTG ACT CTC ACC GAA GTC GAC	943
	Tyr Thr Leu Thr Leu Thr Glu Val Asp	
	85 90	
25	AAC TGG ACC GGA CCT GAT GGC GTC GTC AAG GAG AAG GTC ATG CTG GTT	991
	Asn Trp Thr Gly Pro Asp Gly Val Val Lys Glu Lys Val Met Leu Val	
	95 100 105	
30	AAC GTACGGCACC CCTTTTCTTG TCCTAGGATC TGGGTGATGT GCGTCGTTGC	1044
	Asn	
	CCCTGAGAGA GACTGACCGA GCCTTTGGCT GCAG AAT AGT ATA ATC GTAATTAATT	1100
	Asn Ser Ile Ile	
	110	
35	ATACCGCCCT GCCTCCAGCA GCCCCAGCAG CTCGAGAAGG GTATCTGAAG TTAGTCAGGC	1160
	CTGCTGACCT GACCGGGGCC AACCCACCAT AG GGA CCA ACA ATC TTT GCG GAC	1213
	Gly Pro Thr Ile Phe Ala Asp	
	115	
40	TGG GGC GAC ACG ATC CAG GTA ACG GTC ATC AAC AAC CTC GAG ACC AAC	1261
	Trp Gly Asp Thr Ile Gln Val Thr Val Ile Asn Asn Leu Glu Thr Asn	
	120 125 130 135	
45	GGC GTATGTCTGC TGCTTGCTCT CTTGCTCTCC TCGTCCGCGA CTAATAATAA	1314
	Gly	
50	TATCAACTCG TGTGGAAAAC AG ACG TCG ATC CAC TGG CAC GGA CTG CAC CAG	1366
	Thr Ser Ile His Trp His Gly Leu His Gln	
	140 145	
55	AAG GGC ACC AAC CTG CAC GAC GGC GCC AAC GGT ATC ACC GAG TGC CCG	1414
	Lys Gly Thr Asn Leu His Asp Gly Ala Asn Gly Ile Thr Glu Cys Pro	
	150 155 160	
60	ATC CCG CCC AAG GGA GGG AGG AAG GTG TAC CGG TTC AAG GCT CAG CAG	1462
	Ile Pro Pro Lys Gly Gly Arg Lys Val Tyr Arg Phe Lys Ala Gln Gln	
	165 170 175	
	TAC GGG ACG AGC TGG TAC CAC TCG CAC TTC TCG GCC CAG TAC GGC AAC	1510
	Tyr Gly Thr Ser Trp Tyr His Ser His Phe Ser Ala Gln Tyr Gly Asn	
	180 185 190	
65	GGC GTG GTC GGG GCC ATT CAG ATC AAC GGG CCG GCC TCG CTG CCG TAC	1558
	Gly Val Val Gly Ala Ile Gln Ile Asn Gly Pro Ala Ser Leu Pro Tyr	
	195 200 205 210	

	GAC ACC GAC CTG GGC GTG TTC CCC ATC AGC GAC TAC TAC TAC AGC TCG	1606
	Asp Thr Asp Leu Gly Val Phe Pro Ile Ser Asp Tyr Tyr Tyr Ser Ser	
5	215 220 225	
	GCC GAC GAG CTG GTG GAA CTC ACC AAG AAC TCG GGC GCG CCC TTC AGC	1654
	Ala Asp Glu Leu Val Glu Leu Thr Lys Asn Ser Gly Ala Pro Phe Ser	
	230 235 240	
10	GAC AAC GTC CTG TTC AAC GGC ACG GCC AAG CAC CCG GAG ACG GGC GAG	1702
	Asp Asn Val Leu Phe Asn Gly Thr Ala Lys His Pro Glu Thr Gly Glu	
	245 250 255	
15	GGC GAG TAC GCC AAC GTG ACG CTC ACC CCG GGC CCG CCG CAC CGC CTG	1750
	Gly Glu Tyr Ala Asn Val Thr Leu Thr Pro Gly Arg Arg His Arg Leu	
	260 265 270	
20	CGC CTG ATC AAC ACG TCG GTC GAG AAC CAC TTC CAG GTC TCG CTC GTC	1798
	Arg Leu Ile Asn Thr Ser Val Glu Asn His Phe Gln Val Ser Leu Val	
	275 280 285 290	
	AAC CAC ACC ATG ACC ATC ATC GCC GCC GAC ATG GTG CCC GTC AAC GCC	1846
	Asn His Thr Met Thr Ile Ile Ala Ala Asp Met Val Pro Val Asn Ala	
	295 300 305	
25	ATG ACG GTC GAC AGC CTC TTC CTC GGC GTC GGC CAG CGC TAC GAT GTC	1894
	Met Thr Val Asp Ser Leu Phe Leu Gly Val Gly Gln Arg Tyr Asp Val	
	310 315 320	
30	GTC ATC GAA GCC AGC CGA ACG CCC GGG AAC TAC TGG TTT AAC GTC ACA	1942
	Val Ile Glu Ala Ser Arg Thr Pro Gly Asn Tyr Trp Phe Asn Val Thr	
	325 330 335	
35	TTT GGC GGC GGC CTG CTC TGC GGC GGC TCC AGG AAT CCC TAC CCG GCC	1990
	Phe Gly Gly Gly Leu Leu Cys Gly Gly Ser Arg Asn Pro Tyr Pro Ala	
	340 345 350	
40	GCC ATC TTC CAC TAC GCC GGC GCC CCC GGC GGC CCG CCC ACG GAC GAG	2038
	Ala Ile Phe His Tyr Ala Gly Ala Pro Gly Gly Pro Pro Thr Asp Glu	
	355 360 365 370	
	GGC AAG GCC CCG GTC GAC CAC AAC TGC CTG GAC CTC CCC AAC CTC AAG	2086
	Gly Lys Ala Pro Val Asp His Asn Cys Leu Asp Leu Pro Asn Leu Lys	
	375 380 385	
45	CCC GTC GTG GCC CGC GAC GTG CCC CTG AGC GGC TTC GCC AAG CGG CCC	2134
	Pro Val Val Ala Arg Asp Val Pro Leu Ser Gly Phe Ala Lys Arg Pro	
	390 395 400	
50	GAC AAC ACG CTC GAC GTC ACC CTC GAC ACC ACG GGC ACG CCC CTG TTC	2182
	Asp Asn Thr Leu Asp Val Thr Leu Asp Thr Thr Gly Thr Pro Leu Phe	
	405 410 415	
55	GTC TGG AAG GTC AAC GGC AGC GCC ATC AAC ATC GAC TGG GGC AGG CCC	2230
	Val Trp Lys Val Asn Gly Ser Ala Ile Asn Ile Asp Trp Gly Arg Pro	
	420 425 430	
60	GTC GTC GAC TAC GTC CTC ACG CAG AAC ACC AGC TTC CCA CCC GGG TAC	2278
	Val Val Asp Tyr Val Leu Thr Gln Asn Thr Ser Phe Pro Pro Gly Tyr	
	435 440 445 450	
	AAC ATT GTC GAG GTG AAC GGA GCT GAT CAG GTAAGAAAAA GGGGACCGCA	2328
	Asn Ile Val Glu Val Asn Gly Ala Asp Gln	
	455 460	
65	GGGGTGCTGC TGCAAGTACA CCTTGCTCGC CCTCTGTTC TTCCTTAATA ACTACCTCCC	2388
	AACCTCCCC CCTAATTAAT TCACTTTAAA GGCCGATCAA GACTGACCGA GCCCCCTCTC	2448

5	TTTGCAG TGG TCG TAC TGG TTG ATC GAG AAC GAT CCC GGC GCA CCT TTC Trp Ser Tyr Trp Leu Ile Glu Asn Asp Pro Gly Ala Pro Phe 465 470	2497
10	ACC CTA CCG CAT CCG ATG CAC CTG CAC GTAAGTTGGA TACATATATA Thr Leu Pro His Pro Met His Leu His 475 480	2544
15	TATATATATA TACATTGCTT TCCTGGCTCG CTCCTTAAA TAAAATTAAA TAACCAAAAA TAACCAAAAA AAG GGC CAC GAC TTT TAC GTG CTG GGC CGC TCG CCC GAC Gly His Asp Phe Tyr Val Leu Gly Arg Ser Pro Asp 485 490 495	2604 2653
20	GAG TCG CCG GCA TCC AAC GAG CGG CAC GTG TTC GAT CCG GCG CGG GAC Glu Ser Pro Ala Ser Asn Glu Arg His Val Phe Asp Pro Ala Arg Asp 500 505 510	2701
25	GCG GGC CTG CTG AGC GGG GCC AAC CCT GTG CGG CGG GAC GTG ACG ATG Ala Gly Leu Leu Ser Gly Ala Asn Pro Val Arg Arg Asp Val Thr Met 515 520 525	2749
30	CTG CCG GCG TTC GGG TGG GTG GTG CTG GCC TTC CGG GCC GAC AAC CCG Leu Pro Ala Phe Gly Trp Val Val Leu Ala Phe Arg Ala Asp Asn Pro 530 535 540	2797
35	GGC GCC TGG CTG TTC CAC TGC CAC ATC GCC TGG CAC GTC TCG GGC GGC Gly Ala Trp Leu Phe His Cys His Ile Ala Trp His Val Ser Gly Gly 545 550 555	2845
40	CTG GGC GTC GTC TAC CTC GAG CGC GCC GAC GAC CTG CGC GGG GCC GTC Leu Gly Val Val Tyr Leu Glu Arg Ala Asp Asp Leu Arg Gly Ala Val 560 565 570 575	2893
45	TCG GAC GCC GAC GCC GAC GAC CTC GAC CGC CTC TGC GCC GAC TGG CGC Ser Asp Ala Asp Ala Asp Asp Leu Asp Arg Leu Cys Ala Asp Trp Arg 580 585 590	2941
50	CGC TAC TGG CCT ACC AAC CCC TAC CCC AAG TCC GAC TCG GGC CTC AAG Arg Tyr Trp Pro Thr Asn Pro Tyr Pro Lys Ser Asp Ser Gly Leu Lys 595 600 605	2989
55	CAC CGC TGG GTC GAG GAG GGC GAG TGG CTG GTC AAG GCG TGAGCGAAGG His Arg Trp Val Glu Glu Gly Glu Trp Leu Val Lys Ala 610 615 620	3038
	AGGAAAAAGG AAACAAAGAG GGGGGGGGGG GCTAGTTCCT ATTTTGCTT TTTTTTTTTG TTCTTGTCCT TGTGCTGGCG GTTACCCTGG TAAAGGAGAA GGGGGCCCCA AGTTCGAGTG GGTGTGTGAT CGGGTAAATA TTATCAAGAG ATCT	3098 3158 3192

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 620 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Lys Ser Phe Ile Ser Ala Ala Thr Leu Leu Val Gly Ile Leu Thr



	1		5		10		15
	Pro	Ser	Val	Ala	Ala	Ala	Pro
				20			25
	Thr	Pro	Glu	Gln	Arg	Asp	Leu
5	Leu	Val	Pro	Ile	Thr	Glu	Arg
		35				40	
	Gln	Ser	Cys	Asn	Thr	Pro	Ser
		50				55	
10	Asn	Arg	Ala	Cys	Trp	Thr	Asp
				60			
	Asp	Ile	Asn	Thr	Asp	Tyr	Glu
		65				70	
	Val	Asp	Ser	Pro	Asp	Thr	Gly
				75			80
15	Arg	Pro	Tyr	Thr	Leu	Thr	Leu
				85			90
	Val	Asp	Asn	Trp	Thr	Gly	Pro
				95			
	Asp	Gly	Val	Val	Lys	Glu	Lys
			100				105
20	Leu	Val	Asn	Asn	Ser	Ile	Ile
				110			
	Gly	Pro	Thr	Ile	Phe	Ala	Asp
			115				120
	Trp	Gly	Asp	Thr	Ile	Gln	Val
						125	
25	Ile	Asn	Asn	Leu	Glu	Thr	Asn
		130					135
	Gly	Thr	Ser	Ile	His	Trp	His
				140			
	His	Gln	Lys	Gly	Thr	Asn	Leu
				145			150
	His	Asp	Gly	Ala	Asn	Gly	Ile
				155			160
30	Cys	Pro	Ile	Pro	Pro	Lys	Gly
				165			170
	Val	Tyr	Arg	Phe	Lys	Ala	Gln
				175			
	Gln	Gln	Tyr	Gly	Thr	Ser	Trp
				180			185
35	His	Ser	His	Phe	Ser	Ala	Gln
				190			
	Gly	Asn	Gly	Val	Val	Gly	Ala
			195				200
	Ile	Gln	Ile	Asn	Gly	Pro	Ala
						205	
40	Pro	Tyr	Asp	Thr	Asp	Leu	Gly
						210	215
	Val	Phe	Pro	Ile	Ser	Asp	Tyr
						220	
	Ser	Ser	Ala	Asp	Glu	Leu	Val
						225	230
	Glu	Leu	Thr	Lys	Asn	Ser	Gly
				235			240
45	Phe	Ser	Asp	Asn	Val	Leu	Phe
						245	250
	Thr	Ala	Lys	His	Pro	Glu	Thr
						255	
	Gly	Glu	Gly	Glu	Tyr	Ala	Asn
				260			265
50	Leu	Thr	Pro	Gly	Arg	Arg	His
						270	
	Arg	Leu	Arg	Leu	Ile	Asn	Thr
						275	280
	Ser	Val	Glu	Asn	His	Phe	Gln
						285	
55	Leu	Val	Asn	His	Thr	Met	Thr
						290	295
	Ile	Ile	Ala	Ala	Asp	Met	Val
						300	
	Asn	Ala	Met	Thr	Val	Asp	Ser
						305	310
	Leu	Phe	Leu	Gly	Val	Gly	Gln
				315			320
60	Asp	Val	Val	Ile	Glu	Ala	Ser
						325	330
	Thr	Pro	Gly	Asn	Tyr	Trp	Phe
						335	
	Val	Thr	Phe	Gly	Gly	Gly	Leu
						340	345
	Cys	Gly	Gly	Ser	Arg	Asn	Pro
						350	
65	Pro	Ala	Ala	Ile	Phe	His	Tyr
						355	360
	Gly	Ala	Pro	Gly	Gly	Pro	Pro
						365	


	Asp	Glu	Gly	Lys	Ala	Pro	Val	Asp	His	Asn	Cys	Leu	Asp	Leu	Pro	Asn	
	370						375					380					
5	Leu	Lys	Pro	Val	Val	Ala	Arg	Asp	Val	Pro	Leu	Ser	Gly	Phe	Ala	Lys	
	385					390					395					400	
	Arg	Pro	Asp	Asn	Thr	Leu	Asp	Val	Thr	Leu	Asp	Thr	Thr	Gly	Thr	Pro	
					405					410					415		
10	Leu	Phe	Val	Trp	Lys	Val	Asn	Gly	Ser	Ala	Ile	Asn	Ile	Asp	Trp	Gly	
				420					425					430			
	Arg	Pro	Val	Val	Asp	Tyr	Val	Leu	Thr	Gln	Asn	Thr	Ser	Phe	Pro	Pro	
			435					440					445				
15	Gly	Tyr	Asn	Ile	Val	Glu	Val	Asn	Gly	Ala	Asp	Gln	Trp	Ser	Tyr	Trp	
	450					455						460					
20	Leu	Ile	Glu	Asn	Asp	Pro	Gly	Ala	Pro	Phe	Thr	Leu	Pro	His	Pro	Met	
	465					470					475					480	
	His	Leu	His	Gly	His	Asp	Phe	Tyr	Val	Leu	Gly	Arg	Ser	Pro	Asp	Glu	
					485					490					495		
25	Ser	Pro	Ala	Ser	Asn	Glu	Arg	His	Val	Phe	Asp	Pro	Ala	Arg	Asp	Ala	
				500					505					510			
	Gly	Leu	Leu	Ser	Gly	Ala	Asn	Pro	Val	Arg	Arg	Asp	Val	Thr	Met	Leu	
		515						520					525				
30	Pro	Ala	Phe	Gly	Trp	Val	Val	Leu	Ala	Phe	Arg	Ala	Asp	Asn	Pro	Gly	
	530						535					540					
35	Ala	Trp	Leu	Phe	His	Cys	His	Ile	Ala	Trp	His	Val	Ser	Gly	Gly	Leu	
	545					550					555					560	
	Gly	Val	Val	Tyr	Leu	Glu	Arg	Ala	Asp	Asp	Leu	Arg	Gly	Ala	Val	Ser	
					565					570					575		
40	Asp	Ala	Asp	Ala	Asp	Asp	Leu	Asp	Arg	Leu	Cys	Ala	Asp	Trp	Arg	Arg	
				580					585					590			
	Tyr	Trp	Pro	Thr	Asn	Pro	Tyr	Pro	Lys	Ser	Asp	Ser	Gly	Leu	Lys	His	
			595					600					605				
45	Arg	Trp	Val	Glu	Glu	Gly	Glu	Trp	Leu	Val	Lys	Ala					
	610						615					620					

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13 bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>13</u> , line <u>4-13</u>	
B. IDENTIFICATION OF <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depository institution Agricultural Research Service Patent Culture Collection (NRRL)	
Address of depository institution (including postal code and country)  Northern Regional Research Center 1815 University Street Peoria, IL 61604, US	
Date of deposit 25 May 1994	Accession Number NRRL B-21261
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
In respect of those designations in which a European and/or Australia Patent is sought, during the pendency of the patent application, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC/Regulation 3.25 of Australia Statutory Rule 1991 No. 71).	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indication listed below will be submitted to the International Bureau Later (specify the general nature of the indications e.g. "Accession Number of Deposit")          	

For receiving Office use only

<input checked="" type="checkbox"/> This sheet was received with the international application
Authorized officer 

For International Bureau use only

<input type="checkbox"/> This sheet was received with the International Bureau on:
Authorized officer

## PATENT CLAIMS

1. A dyeing composition comprising
  - a) above 0 to 1 mg enzyme protein per ml dyeing composition of  
5 microbial laccase,
  - b) one or more dye precursor, and
  - c) optionally one or more dye modifiers.
2. The dyeing composition according claims 1, wherein the laccase is presents in a concentration of from 0.0001 to 1  
10 mg/ml, preferably 0.001 to 0.8 mg/ml, more preferred 0.002 to 0.5 mg/ml, even more preferred 0.003 to 0.2 mg/ml, especially 0.004 to 0.1 mg enzyme protein/ml dyeing composition.
3. The dyeing composition according to claims 1 and 2, wherein said microbial laccase is of filamentous fungus origin.
- 15 4. The dyeing composition according to claims 1 and 2, wherein the laccase is derived from a strain of the genus *Myceliophthora*, in particular a strain of species *Myceliophthora thermophila*, such as *Myceliophthora thermophila* NRRL B 21261, or variants thereof, such as the T1 variant.
- 20 5. The dyeing composition according to claim 4, wherein the laccase is encoding by the sequence shown in SEQ ID NO 1.
6. The dyeing composition according to claims 4 and 5, wherein the laccase is present in a concentration of from above 0 to 1 mg/ml, preferably 0.0001 to 0.1 mg/ml, more preferably  
25 0.0005 to 0.05 mg/ml, especially 0.001 to 0.01 mg enzyme protein/ml dyeing composition.
7. The dyeing composition according to any of claims 1 to 6, comprising a dye precursor selected from the group comprising  
30 p-phenylene-diamine (PPD), p-toluylene-diamine (pTD), chloro-p-phenylenediamine, p-aminophenol, o-aminophenol, 3,4-diaminotoluene, 2-methyl-1,4-diaminobenzene, 4-methyl-o-phenylenediamine, 2-methoxy-p-phenylenediamine, 2-chloro-1,4-diamino-benzene, 4-amino diphenylamine, 1-amino-4- $\beta$ -methoxyethylamino-benzene, 1-amino-4-bis-( $\beta$ -hydroxyethyl)-amonibenzene, 1-3-diamino-  
35 benzene, 2-methyl-1,3-diamino-benzene, 2,4-diaminotoluene, 2,6-diaminopyridine, 1-hydroxy-2-amino-benzene, 1-hydroxy-3-amino-benzene, 1-methyl-2-hydroxy-4-amino-benzene, 1-methyl-2-hydro-

xy-4- $\beta$ -hydroxyethylamino-benzene, 1-hydroxy-4-amino-benzene, 1-hydroxy-4-methylamino-benzene, 1-methoxy-2,4-diamino-benzene, 1-ethoxy-2,3-diamino-benzene, 1- $\beta$ -hydroxyethyloxy-2,4-diamino-benzene, phenazines, such as 4,7-phenazinedicarboxylic acid, 5 2,7-phenazinedicarboxylic acid, 2-phenazinecarboxylic acid, 2,7-diaminophenazine, 2,8-diaminophenazine, 2,7-diamino-3,8-dimethoxyphenazine, 2,7-diamino-3-methoxyphenazine, 2,7-diamino-3-methoxyphenazine, 3-dimethyl 2,8-phenazinediamine, 2,2'-[(8-amino-7-methoxy-2-phenazinyl)imino]bis-ethanol, 2,2'-[(8-amino-7-methoxy-2-phenazinyl)imino]bis-ethanol, 2,2'-[(8-amino-7-chloro-2-phenazinyl)imino]bis-ethanol, 2-[(8-amino-7-methyl-2-phenazinyl)amino]-ethanol, 2,2'-[(8-amino-2-phenazinyl)imino]-bis-ethanol, 3-amino-7-(dimethylamino)-2,8-dimethyl-5-phenyl-chloride, 9-(diethylamino)- benzo[a]phenazine-1,5-diol, N-[8- 15 (diethylamino)-2-phenazinyl]- methanesulfonamide, N-(8-methoxy-2-phenazinyl)-methanesulfonamide, N,N,N',N'-tetramethyl-2,7-phenazinediamine, 3,7-dimethyl-2-phenazinamine, p-amino benzoic acids, such as p-amino benzoic acid ethyl, p-amino benzoic acid glycerid, p-amino benzoic acid isobutyl, p-dimethylamino 20 benzoic acid amil, p-dimethylamino benzoic acid octyl, p-diethoxy amino benzoic amil, p-dipropoxy amino benzoic acid ethyl, acetylsalicylic acid, isatin derivatives, such as 2,3-diamino benzoic acid.

8. The dyeing composition according to any of claims 1 to 7, 25 comprising a dye modifier selected from the group comprising m-phenylene-diamine, 2,4-diaminoanisole, 1-hydroxynaphthalene( $\alpha$ -naphthol), 1,4-dihydroxybenzene(hydroquinone), 1,5-dihydroxynaphthalene, 1,2-dihydroxybenzene(pyrocatechol), 1,3-dihydroxybenzene (resorcinol), 1,3-dihydroxy-2-methylbenzene, 1,3-dihydroxy-4-chlorobenzene (4-chlororesorcinol), 1,2,3,trihydroxybenzene, 1,2,4-trihydroxybenzene, 1,2,4-trihydroxy-5-methylbenzene, 1,2,4-trihydroxytoluene. 30

9. Use of the composition according to claim 1 to 8 for permanent dyeing of keratinous fibres, such as hair, fur, hide 35 or wool.

10. A method for dyeing keratinous fibres comprising contacting a dyeing composition according to claims 1 to 8 to

the keratinous fibres under suitable conditions and for a period of time sufficient to permit oxidation of the dye precursor into a coloured compound.

11. The method according to claim 10, wherein the dyeing  
5 procedure is carried out at a pH in the range from 3 to 10, preferably 5 to 9, especially 6 to 8.

12. The method wherein according to claims 10 and 11,  
wherein the procedure is carried out for a period of time  
between 10 and 60 minutes, preferably 15 to 50 minutes,  
10 especially 20 to 40 minutes.

1/4

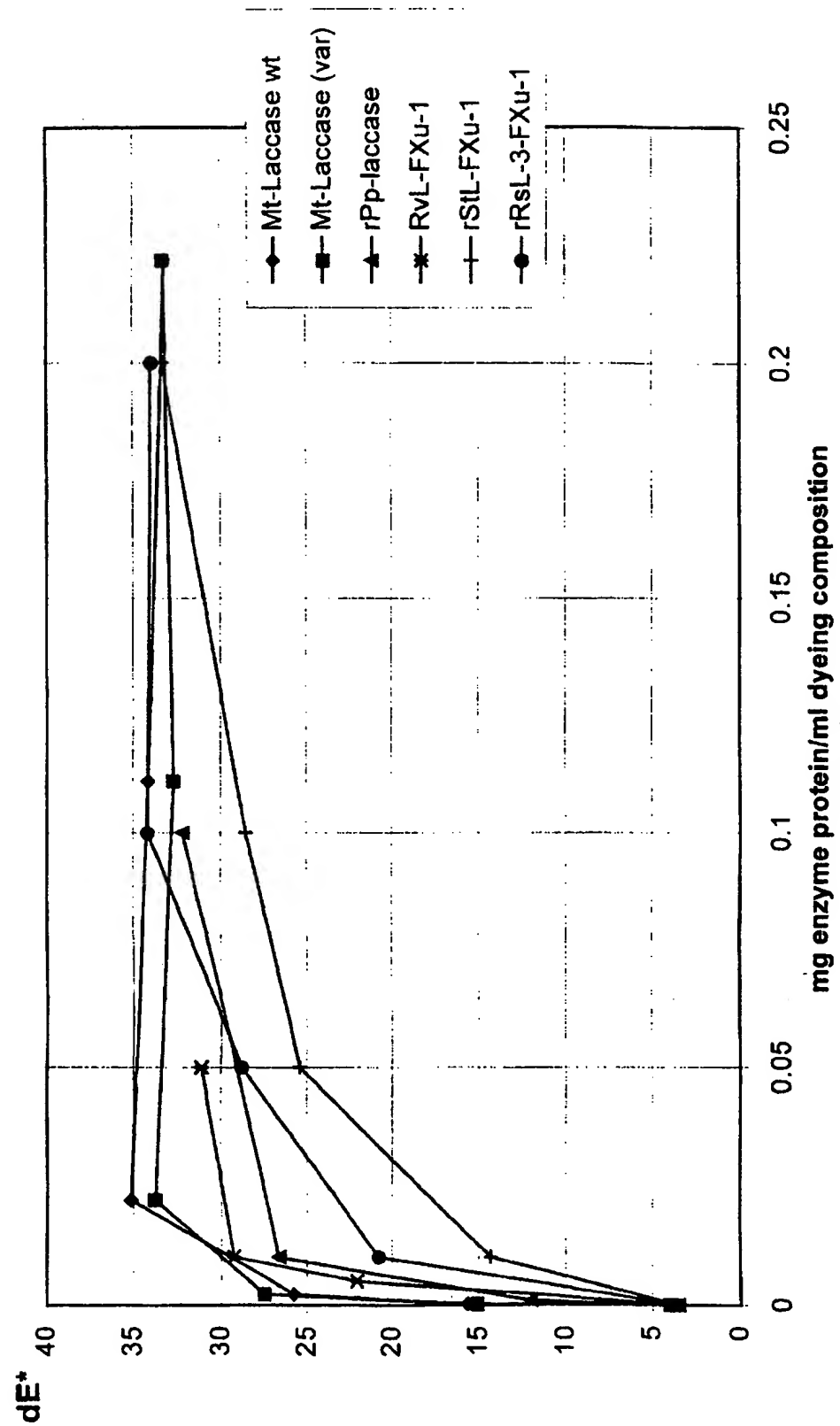
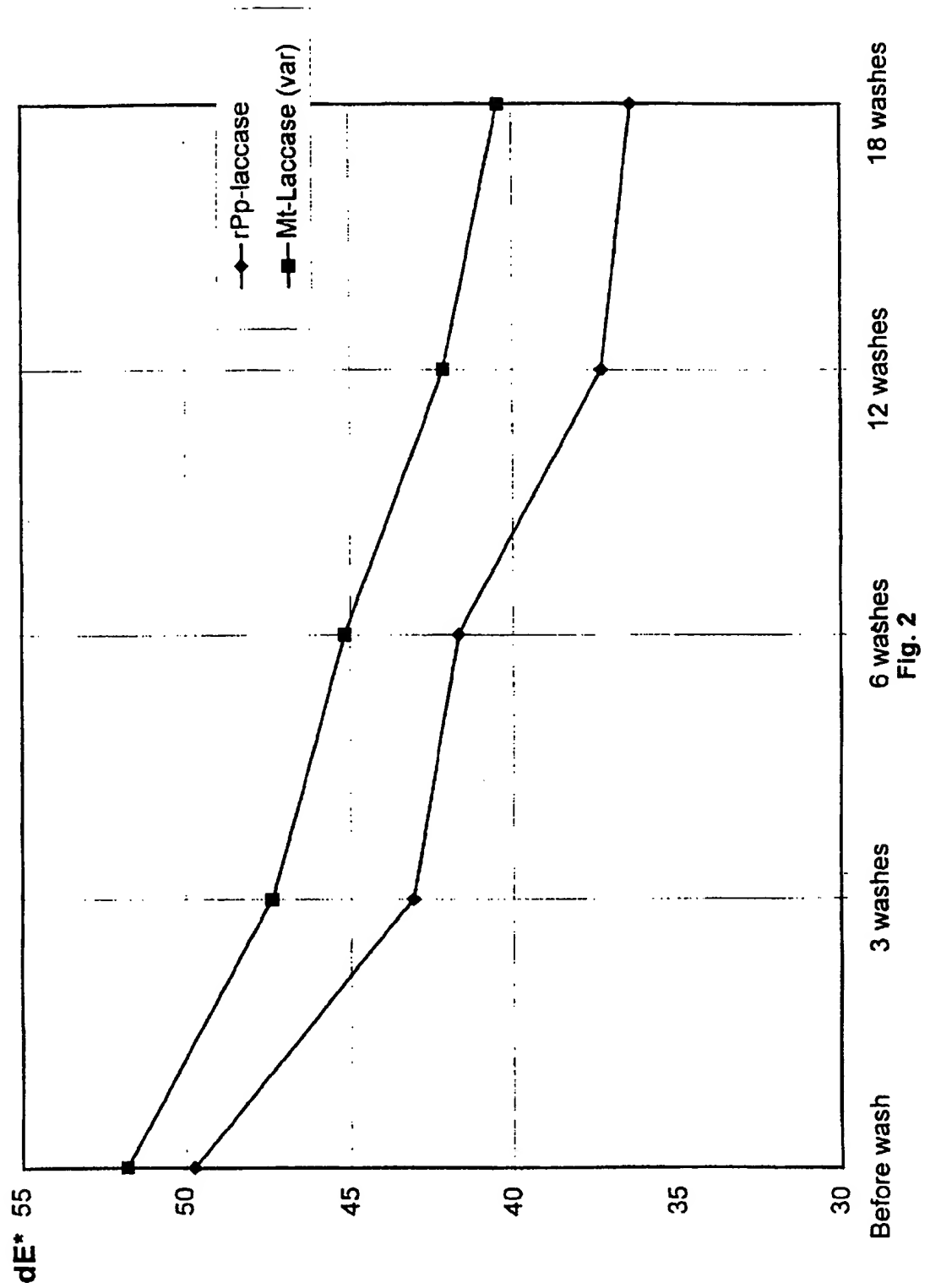


Fig. 1

2/4





3/4

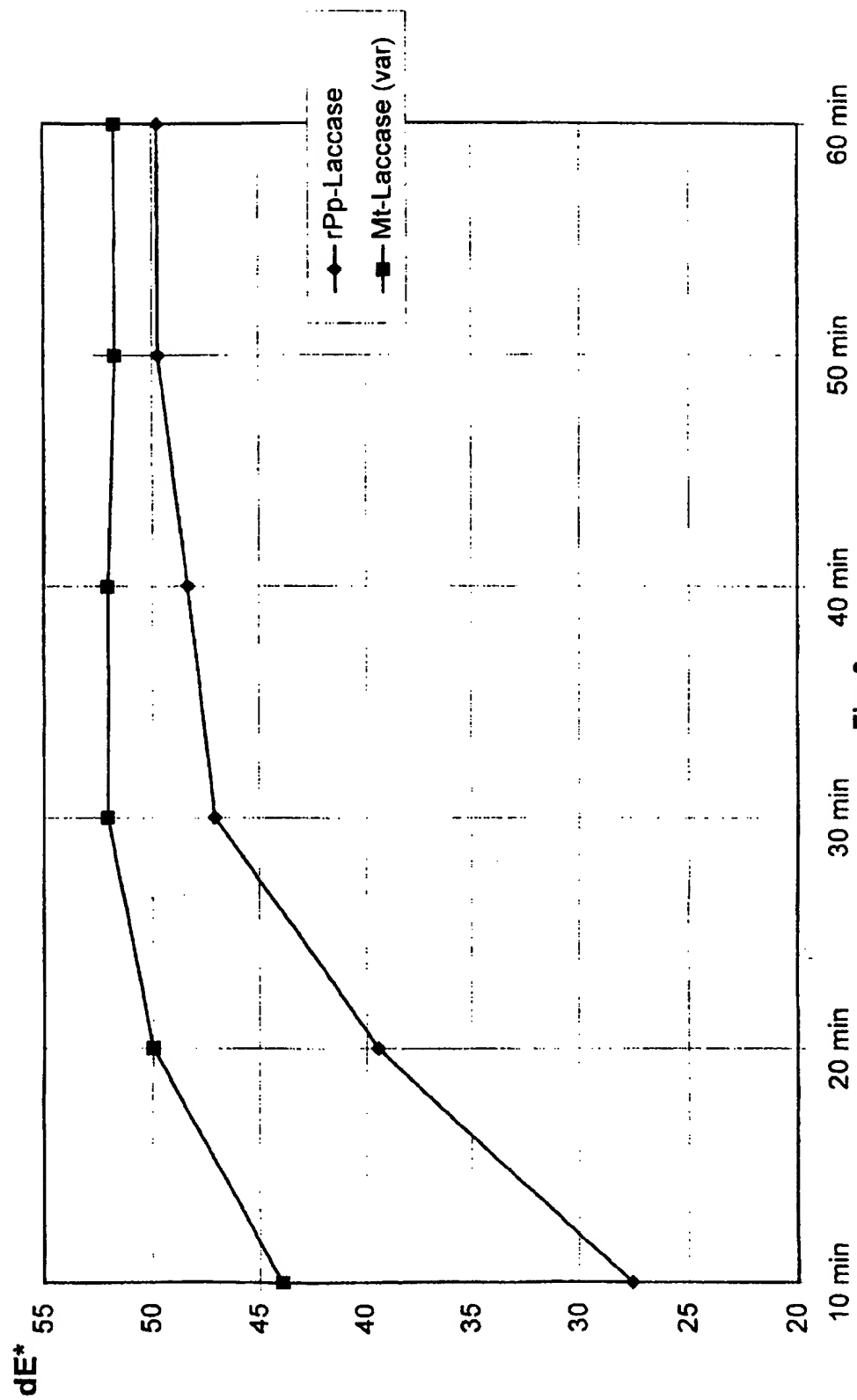
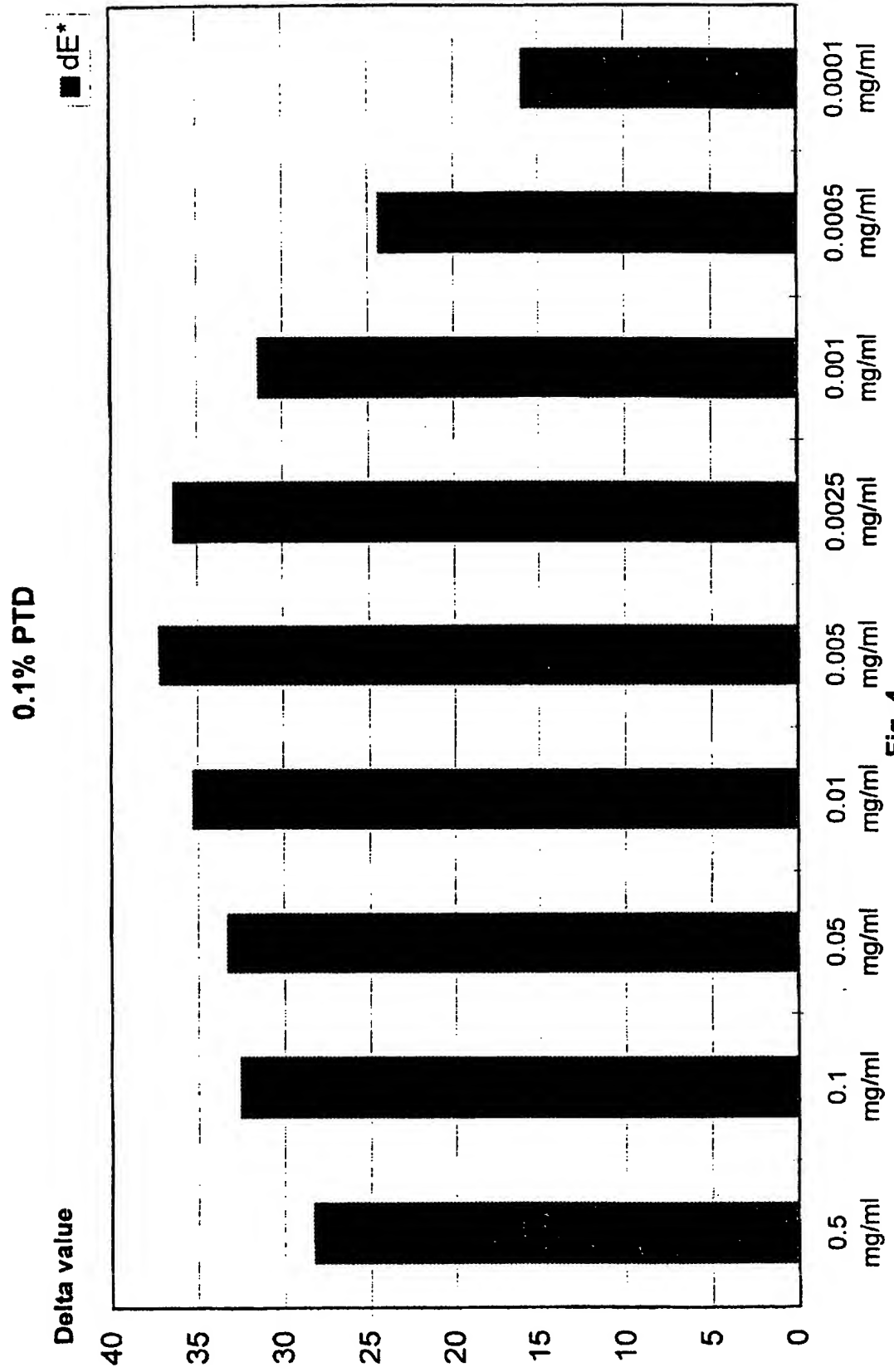


Fig. 3

4/4



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 96/00499

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C09B 67/00, A61K 7/13

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C09B, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 9533836 A1 (NOVO NORDISK BIOTECH, INC.), 14 December 1995 (14.12.95), claims 31-42; page 16, line 12 - page 17, line 27; page 34, line 20 - page 36 --	1-12
P,X	WO 9533837 A1 (NOVO NORDISK BIOTECH, INC.), 14 December 1995 (14.12.95), claims 28, 29; page 15, line 34 - page 16, line 2 --	1-3
P,A	--	4-12
X	EP 0504005 A1 (PERMA SOCIETE ANONYME), 16 Sept 1992 (16.09.92) --	1-3
A	--	4-12

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

## \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

28 February 1997

Date of mailing of the international search report

01 -03- 1997

Name and mailing address of the ISA/

Swedish Patent Office

Box 5055, S-102 42 STOCKHOLM

Facsimile No. +46 8 666 02 86

Authorized officer

Gerd Strandell

Telephone No. +46 8 782 25 00

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 96/00499

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9600290 A1 (NOVO NORDISK BIOTECH, INC.), 4 January 1996 (04.01.96), claims 37-48; page 48, line 25 - page 54, line 24	1-3
A	--	4-12
X	STN International, File CAPLUS, CAPLUS accession no. 1991:498981, Saruno, Rinjiro: "Hair- dyeing preparations containing melanin or other poly- phenol pigments and manufacture of the pigments"; & JP,A,03077813, 910403	1-3
X	STN International, File CAPLUS, CAPLUS accession no. 1995:974547, Chivukula, Muralikrishna et al: "Phenolic azo dye oxidation by laccase from Pyricularia oryzae"; & Appl. Environ. Microbiol. (1995), 61(12), 4374-77	1-3
A	US 3251742 A (SAUL SOLOWAY), 17 May 1966 (17.05.66)	1-12
A	WO 9507988 A1 (NOVO NORDISK A/S), 23 March 1995 (23.03.95), claim 41	1-12
A	DE 4314317 A1 (HENKEL KGAA), 3 November 1994 (03.11.94)	7
A	WO 9400100 A1 (L'OREAL), 6 January 1994 (06.01.94)	7

INTERNATIONAL SEARCH REPORT  
Information on patent family members

03/02/97

International application No.  
PCT/DK 96/00499

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9533836	14/12/95	AU-A- 2656595	04/01/96
WO-A1- 9533837	14/12/95	AU-A- 2656695	04/01/96
EP-A1- 0504005	16/09/92	AT-T- 121931	15/05/95
		CA-A- 2061826	09/09/92
		DE-D,T- 69202290	09/11/95
		ES-T- 2072720	16/07/95
		FR-A,B- 2673534	11/09/92
		JP-A- 6172145	21/06/94
WO-A1- 9600290	04/01/96	AU-A- 2827895	19/01/96
US-A- 3251742	17/05/66	FR-A- 1363462	00/00/00
		GB-A- 993923	00/00/00
WO-A1- 9507988	23/03/95	AU-A- 7833694	03/04/95
		CA-A- 2171288	23/03/95
		CN-A- 1133067	09/10/96
		EP-A- 0719337	03/07/96
		FI-A- 961250	18/03/96
		US-A- 5480801	02/01/96
DE-A1- 4314317	03/11/94	EP-A- 0695162	07/02/96
		JP-T- 8509478	08/10/96
		WO-A- 9424988	10/11/94
WO-A1- 9400100	06/01/94	DE-D,T- 69301464	05/06/96
		EP-A,B- 0645999	05/04/95
		FR-A,B- 2692782	31/12/93
		JP-T- 7508271	14/09/95
		US-A- 5538517	23/07/96